Summary Report

Peer Review of EPA's Draft Document

Health Effects Support Document for the Cyanobacterial Toxin Cylindrospermopsin

September 29, 2014

Peer Reviewers:

Ian R. Falconer, Ph.D.
James S. Metcalf, Ph.D.
Brett A. Neilan, Ph.D.

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Contents

I. INTRODUCTION

Cyanobacteria, also known as blue-green algae, are commonly found in fresh, estuarine, and marine waters in the United States. They are of special concern because they can produce highly potent cyanobacterial toxins that could cause health effects, especially in the liver, nervous system, and the skin. Because of increasing public health concerns about cyanobacterial toxins in drinking waters, EPA has decided to develop a Drinking Water Health Advisory for cylindrospermopsin.

Drinking Water Health Advisories (DWHA) serve as the informal technical guidance to assist EPA's Regional Offices, state and local officials, and managers of public or community water systems on health effects, analytical methods, and treatment technologies associated with unregulated drinking water contaminants. Health Advisories are guidance values based on noncancer health effects for different durations of exposure (e.g., one-day, ten-day, and lifetime). They are not to be construed as legally enforceable federal standards and they are subject to revision as new information becomes available. EPA has developed the *Health Effects Support Document for the Cyanobacterial Toxin Cylindrospermopsin* as the scientific basis for the development of the DWHA for cylindrospermopsin.

The purpose of this peer review is for EPA to receive written comments from three scientific experts on the document.

Peer Reviewers :

Ian R. Falconer, Ph.D.
Professor Emeritus
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Brett A. Neilan, Ph.D.
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II. CHARGE TO REVIEWERS

The peer reviewers charge questions are divided by Chapters and General Questions as follows:

Chapters 2, 5, and 6 of the Health Effects Support Document provide information on the chemical and physical properties, exposure, and the toxicokinetics of cylindrospermopsin.

1. Are you aware of any additional data that should be included in the document? If so, please provide.
2. Is any of the information included in the document or conclusions incorrect, redundant or irrelevant? Please describe.
3. Please comment on the flow and continuity of these chapters and provide suggestions to enhance the utility of these chapters, if needed.

Chapter 7 - Hazard Identification. This chapter outlines the toxicity studies and the epidemiology, genotoxicity and mechanistic data. This chapter also includes the characterization of human health effects.

1. Are you aware of any additional critical studies for cylindrospermopsin that should be included in the document? If so, please provide.
2. Is any of the information included in the document incorrect, redundant or irrelevant? Please describe and provide suggestions, if needed.
3. Are the conclusions and critical discussions for cylindrospermopsin valid and scientifically defensible? Please describe and provide suggestions, if needed.

Chapter 8 - Dose-Response Assessment. This chapter provides the dose-response assessment and the derivation of RfDs.

A. Data sufficiency

    1. Is the conclusion that there are sufficient data to derive a reference dose (RfD) for cylindrospermopsin adequately justified? Please describe and provide suggestions, if needed.
    2. Have critical data gaps been identified and/or addressed for cylindrospermopsin? Please describe and provide suggestions, if needed.

B. Identification of the critical study
   The Humpage and Falconer study (2002, 2003) was selected as the critical study for derivation of the RfD for cylindrospermopsin.

        1. Is the study methodology sound? Please describe and provide suggestions, if needed.
        2. Are strengths and weaknesses of the study and the accompanying mode of action appropriately described? Please provide suggestions, if needed.

3. Do the results of this study represent the best available science and most appropriate toxicological endpoint for the basis of an oral RfD for cylindrospermopsin?

C. Calculation of RfD

This Health Effects Support Document proposes an oral RfD for cylindrospermopsin based on the kidney weight data from the Humpage and Falconer studies (2002 and 2003) and supported by the hematological results identified in the Sukenik et al. 2006 study, and Reisner et al. 2004 (attached).

1. Is the calculation of the RfD for cylindrospermopsin clear and accurate? Please describe and provide suggestions, if needed.
2. Has uncertainty (via uncertainty factors) been adequately accounted for in the derivation of the RfD? Please describe and provide suggestions, if needed.

Chapter 9 – Research Gaps.

1. Have critical data gaps been identified and/or addressed for cylindrospermopsin? Please describe and provide suggestions, if needed.

General Questions

1. Is the document clear and understandable? Please describe and provide suggestions, if needed.
2. Are you aware of any additional data that should be addressed in the document? If so, please provide a reference.
3. Are you aware of any additional issues that should be addressed in the document? If so, please describe.

III. PEER REVIEW COMMENT TABLE

I. GENERAL IMPRESSIONS		
NAME	**COMMENT**	**RESPONSE**
Ian R. Falconer, Ph.D.	This is a comprehensive and well assembled review of the field of cylindrospermopsin occurrence and toxicology. It is overall an accurate account of the available data, and the interpretation of the available information. Some specific points will be raised later, but there are no significant errors. The presentation is systematic, but as a consequence of the approach, there is overlap of content. This can be consolidated, but the comprehensiveness of individual sections when viewed alone would be lost. If it is intended that chapters can be viewed as entities without reference to other chapters, then this comment can be ignored. The conclusions are sound. The data from which the conclusions are drawn have a good level of internal consistency, with research groups from different laboratories, and countries, contributing to the overall conclusions. The issue of carcinogenicity of cylindrospermopsin will have to be addressed at more length when the crucial long-term carcinogenicity studies have been undertaken. Unfortunately, the cost of these studies has prevented their initiation up to the present time, and the focus, by necessity, has been on short-term and *in-vitro* research.	
James S. Metcalf, Ph.D.	The EPA document "Health Effects Support Document for Cyanobacterial Toxin Cylindrospermopsin" is an accurate, clear document addressing the effects of cylindrospermopsin in cells and mammals. Although it is clear and sound, there is a possibility to improve the document further, in order to maintain up to date and current information concerning the occurrence and toxicity of cylindrospermopsin and its variants. For example, in addition to cylindrospermopsin, 7-epicylindrospermopsin and deoxycylindrospermopsin, new variants of cylindrospermopsin are being isolated and characterized (desulfo-cylindrospermopsin variants, Wimmer et al., 2014; Harmful Algae 37: 203-206), which although no toxicity data is available, do require mention, as they may contribute to the overall toxicity of cylindrospermopsin variants present in blooms and strains of cyanobacteria. Furthermore, although difficult, a large number of studies have examined the effects of cylindrospermopsin by use of extracts of cyanobacteria, with the possibility of synergistic effects with other bioactive compounds that may be present. It may be useful for strengthening the document by separating toxicity studies using extracts from	

	those with purified cylindrospermopsin variants, along with some discussion concerning the different toxicity outcomes between such studies. In addition, it may be useful to include some invertebrate or plant based toxicity assays for risk assessment. This is because useful information concerning adverse cylindrospermopsin effects can be ascertained. For example, Lindsay et al. (2006; Toxicon 48: 995-1001) showed that co-exposure of LPS and cylindrospermopsin affected the subsequent LC_{50} values in brine shrimp, which has direct relevance for assessing human exposures to cylindrospermopsin in cyanobacterial blooms. However, the document as presented, does provide an accurate, up to date current risk assessment of cylindrospermopsin, based on current knowledge.	
Brett A. Neilan, Ph.D.	Overall, the information in the document is accurate and clearly presented. The final conclusions are sound, however the manuscript could be improved by further critical evaluation of the literature throughout. The main flaws of this manuscript are the inconsistent level of detail and the general lack of synthesis of the toxicological studies cited. For example, Chapters 2-5 contain a large volume of superfluous and very general information on cyanobacteria and should be streamlined to include information specific to cylindrospermopsin and its producers. In contrast, Chapters 6-7, dealing with cynlindrospermopsin toxicology, contain very detailed information but lack synthesis and a critical review of this data. These omissions are partially addressed in Section 7.6, however, the brief summary paragraphs provided are inadequate and the disjointed structure employed disrupts the flow of the manuscript, which then necessitates repetition of the information.	

II. RESPONSE TO CHARGE QUESTIONS		
Chapters 2, 5, and 6 of the Health Effects Support Document provide information on the chemical and physical properties, exposure, and the toxicokinetics of cylindrospermopsin.		
CHARGE QUESTION 1: ***Are you aware of any additional data that should be included in the document? If so, please provide.***		
NAME	**COMMENT**	**RESPONSE**
Ian R. Falconer, Ph.D.	Chapter 2.0, Page 13. Some comment on the high likelihood of raised DOC coincident with cyanobacterial blooms will be of value. In general, it is when water treatment plants are 'swamped' with organic material from a cyanobacterial bloom that toxins get through into the supply. See: "Evidence of liver damage by toxin from a bloom of the blue-green alga Microcystis aeruginosa". Ian R. Falconer, Arthur M. Beresford, and Maria T.C. Runnegar; Med.J.Aust, 1983, 1: 511-514. Chapter 4.0, Page 14, Section 4.1, Paragraph 1. Marine cyanobacteria grow as benthic organisms in shallow waters, often well out to sea, as well as free-floating water blooms. The most toxic, Lyngbya, grows below the low tide line. See: "Cyanobacteria and Cyanobacterial Toxins" in Oceans and Human Health, eds. Walsh P J et al., Chapter 15, pp, 271-296. Academic Press, 2008. Section 4.2.2 - drinking water. In the light of the very brief discussion here, reference to "Cyanobacterial Toxins of Drinking Water Supplies: Cylindrospermopsins and Microcystins," Ian Robert Falconer, CRC Press, Boca Raton, 2005, would be appropriate here. This section deserves a much more thorough approach, since it is the key issue for the whole review.	
James S. Metcalf, Ph.D.	It would be useful to include some mention and discussion of the study of Wimmer et al. (2014; Harmful Algae 37: 203-206) as they have described new de-sulfo variants of cylindrospermopsin, which to this reviewer, have not been discussed in prior documents pertaining to the adverse health effects of cylindrospermopsin.	
Brett A. Neilan, Ph.D.	These sections are comprehensive. I am unaware of any additional data that should be included.	

CHARGE QUESTION 2: ***Is any of the information included in the document or conclusions incorrect, redundant or irrelevant? Please describe.***		
NAME	**COMMENT**	**RESPONSE**
Ian R. Falconer, Ph.D.	Section 3.2.1.2, Page 12, Paragraph 3, Lines 5 and 6. This sentence is confusing, mixing the concept of activated carbon efficiency-which is a chemical and measurable property, the dose used, and the problems generated by dissolved organic carbon liberated during water blooms. Suggest expansion and re-writing, using the quoted references as sources.	
James S. Metcalf, Ph.D.	There is no incorrect, redundant, or irrelevant information in the document.	
Brett A. Neilan, Ph.D.	Chapter 2 should be streamlined to focus more on cylindrospermopsin-producing cyanobacteria. Chapter 5 contains largely irrelevant information on the occurrence of cyanotoxins in dietary supplements. This section should be condensed since cylindrospermopsin is a potential but non-specific risk. To my knowledge, there have been no reports of cylindrospermopsin contamination in such health products. The negative results of several studies suggest that the risk of cylindrospermopsin poisoning by this route is very low.	
CHARGE QUESTION 3: ***Please comment on the flow and continuity of these chapters and provide suggestions to enhance the utility of these chapters, if needed.***		
NAME	**COMMENT**	**RESPONSE**
Ian R. Falconer, Ph.D.	It is a large topic jump from occurrence of toxin to toxicokinetics. An introductory paragraph in Section 6 would smooth this and lead the reader from one to the other.	
James S. Metcalf, Ph.D.	The chapters flow and continuity are fine.	
Brett A. Neilan, Ph.D.	The flow and continuity of Chapters 2 and 5 are acceptable. Chapter 6 lacks proper structure, that is, there is no introduction/overview or discussion. The chapter is simply a summary of the results of various animal studies. Like most of the other toxicology sections, it reads like a mosaic of scientific abstracts. The reader is left to wade through the data and formulate their own conclusions.	

Chapter 7 - Hazard Identification. This chapter outlines the toxicity studies and the epidemiology, genotoxicity and mechanistic data. This chapter also includes the characterization of human health effects.		
CHARGE QUESTION 1: ***Are you aware of any additional critical studies for cylindrospermopsin that should be included in the document? If so, please provide.***		
NAME	**COMMENT**	**RESPONSE**
Ian R. Falconer, Ph.D.	Section 7.1, Paragraphs 1 and 2. There are much more clinical data, but it is not published due to confidentiality provisions and the sensitive nature of the population, which was about 2,000 aboriginal persons, most of whom were exposed to the dam water supply. Preliminary examination of the cancer rates over the period 1982-1999, comparing the Palm Island population with comparable aboriginal population elsewhere, showed raised frequency of gastrointestinal, kidney and liver cancers, whereas the total cancer frequency was lower in the Palm Island population (unpublished). Copper sulphate precipitates rapidly from the water column. When used for cyanobacterial control, it is used at 1mg/L in the top metre of water, to provide an effective concentration of about 60 micrograms/L cupric ion. This has been measured to disappear within 60-120 minutes. See "Cyanobacterial Toxins of Drinking Water Supplies: Cylindrospermopsins and Microcystins" Ian Robert Falconer, CRC Press, Boca Raton, 2005. There is no real possibility that the poisoning event was due to copper. Page 24 - Other routes of exposure. The toxicity seen in dialysis patients in Caruaru was probably due to the combined effects of microcystin and cylindrospermopsin, as there was nearly 10 times as much cylindrospermopsin in the filters as microcystin. From my observation of the human liver histopathology, it was not possible to draw a finite conclusion, though it was not similar to microcystin damage seen in mice. Page 28 - A relevant paper on reproductive and foetal toxicity is "Oral exposure to cylindrospermopsin in pregnant rats": Reproduction and Foetal Toxicity studies, Almeida et al., 2013, Toxicon 74: 127-129. The results support the other studies, and conclude that the drinking water guideline value for cylindrospermopsin is safe (while they only quote proposed guidelines, these have been adopted with small variation, in Brazil, Australia, Canada).	

	Page 30, last paragraph, last line. Re–examination of the original data for the report refers to mice C1M2 and C1M3 in the 240ug/kg/day dose group as having pathological changes in the kidney. Mouse C1M3, with the small groups of degenerating proximal tubule cells with leucocyte infiltration, had the highest kidney weight of any animal in the whole trial. Thus, the statement on page 59 of the report (ambiguously) refers to two mice with pathological kidney changes, not two sections from the same mouse. Page 36, Paragraphs 1 and 2. The paper by Marie (quoted) concluded that the cell transforming activity of CYN indicates carcinogenic potential at very low concentrations *in vitro*. This with other studies quoted strengthens the case for careful investigation of carcinogenesis by CYN. Page 44, Section 7.5.3. The analysis of the cyanobacterial toxin content of the dialysis filters showed 2.2 ug microcystin per gram of filter material and 19.7 ug/g of cylindrospermopsin. However, there have been no studies on the toxicity of mixtures *in vivo* or *in vitro*. (Carmichael and Azevedo, 2001). Page 45, Section 7.6.1. There is *in vivo* data for ribosomal separation from membranes of the endoplasmic reticulum (Terao, 1994). Page 46, Paragraph 1, last line. The relevance of kidney effects in mice to human injury is very clear! The Palm island poisoning showed the most severe impacts on kidney function; water, electrolytes and protein loss led to the hospitalization of the children and several being put in intensive care, with electrolyte and protein replacement. Sixty nine percent (69%) of the children required intravenous fluids, and 12% required intravenous plasma protein solution. Without this support for kidney malfunction, several would have died. This human data strongly supports the experimental data on the impact of cylindrospermopsin on kidney function. For further *in vivo* mouse toxicology, see also Falconer et al., 1999, Environmental Toxicology, 14:143-150.	
James S. Metcalf, Ph.D.	It may be useful to include some toxicity studies using invertebrates. For example, Lindsay et al. (2006) showed that co-exposure of cyanobacterial LPS and cylindrospermopsin affected the subsequent toxicity outcome, compared to the individual cyanotoxins. Therefore, such studies should be included as they may affect the subsequent human health	

	risk assessment, especially as cyanobacterial LPS is considered to be always present in blooms, in addition to bacterial LPS from associated bacteria.	
Brett A. Neilan, Ph.D.	This section is comprehensive. I am unaware of any additional data that should be included.	
CHARGE QUESTION 2: ***Is any of the information included in the document incorrect, redundant or irrelevant? Please describe and provide suggestions, if needed.***		
NAME	**COMMENT**	**RESPONSE**
Ian R. Falconer, Ph.D.	See comments above.	
James S. Metcalf, Ph.D.	There is no incorrect, redundant or irrelevant information in the document	
Brett A. Neilan, Ph.D.	All the information in Chapter 7 appears to be accurate and relevant.	
CHARGE QUESTION 3: ***Are the conclusions and critical discussions for cylindrospermopsin valid and scientifically defensible? Please describe and provide suggestions, if needed.***		
NAME	**COMMENT**	**RESPONSE**
Ian R. Falconer, Ph.D.	Yes, the overall assessment is correct and defensible.	
James S. Metcalf, Ph.D.	The conclusions and critical discussion for cylindrospermopsin are valid and scientifically defensible.	
Brett A. Neilan, Ph.D.	The conclusions and critical discussions are valid and scientifically defensible. However, further acknowledgement of the limitations of the published studies used to construct this section should be included. The lack of standardization of methods, controls, animal species, toxins, and sample sizes make it difficult to compare these previous studies. This has been a serious problem in cyanotoxin research as in other aspects of environmental toxicology and should be acknowledged in this section of the draft EPA document in order to make any reader/user of the information aware of these limitations.	

Chapter 8 - Dose-Response Assessment. This chapter provides the dose-response assessment and the derivation of RfDs.		
A. Data sufficiency		
CHARGE QUESTION 1: ***Is the conclusion that there are sufficient data to derive a reference dose (RfD) for cylindrospermopsin adequately justified? Please describe and provide suggestions, if needed.***		
NAME	**COMMENT**	**RESPONSE**
Ian R. Falconer, Ph.D.	The data presented show a good level of consistency between research groups and between animal strains. The overall organ sensitivity to the toxin shown in *in vivo* studies demonstrates kidney toxicity as a major concern, which fits with the extensive kidney damage seen in the human population, which was poisoned. Since the kidney weight data are the most sensitive, they are an effective parameter for reference dose calculation. There are sufficient data, backed up by the range of studies presented, and the detailed nature of the key study.	
James S. Metcalf, Ph.D.	In terms of mammals, the authors have used all of the available toxicity data. The only potential issue arises from the fact that only one study used purified CYN, whereas the remainder used cell-free extracts. This latter point may have issues concerning potential synergistic or additive effects from other bioactive compounds present within the extract. However, no other mammalian data are currently available.	
Brett A. Neilan, Ph.D.	The lack of human case studies makes it difficult to assess the effect of cylindrospermopsin on humans. All risk assessments must therefore be based on data obtained from animal experiments. However, relatively few comparable animal toxicology studies have been conducted for cylindrospermopsin. In the present manuscript, the RfD for humans is based on two studies (Humpage and Falconer 2002, 2003). These studies relied on a small sample size (10 mice). The results were statistically significant and the proposed No Observed Adverse Effect Level (NOAEL) for mice is adequately justified. Extrapolating these results to humans is complicated and numerous factors must be considered, including differences in toxin transport and metabolism, as well as relative body size and mode of introduction. It is therefore impossible to accurately predict the Equivalent NOAEL for humans based on these studies alone. Further toxicological and pharmacokinetic animal studies are required to enable accurate scaling and estimation of NOAEL before a confident RfD value can be put forward.	

CHARGE QUESTION 2: ***Have critical data gaps been identified and/or addressed for cylindrospermopsin? Please describe and provide suggestions, if needed.***		
NAME	**COMMENT**	**RESPONSE**
Ian R. Falconer, Ph.D.	There is a major gap in carcinogenicity assessment. No chronic rodent carcinogenicity study has been undertaken, notwithstanding the indicative in vitro research data. The cancer incidence in individuals identified as being hospitalized in the Palm Island poisoning, has not been obtained due to social issues. An overall cancer rate study showed increased cancers in specific organs, but no total increase in this population compared to similar groups.	
James S. Metcalf, Ph.D.	There are no further critical gaps.	
Brett A. Neilan, Ph.D.	Yes. The manuscript identifies the major knowledge gaps for cylindrospermopsin research.	
B. Identification of the critical study		
The Humpage and Falconer study (2002, 2003) was selected as the critical study for derivation of the RfD for cylindrospermopsin.		
CHARGE QUESTION 1: ***Is the study methodology sound? Please describe and provide suggestions, if needed.***		
NAME	**COMMENT**	**RESPONSE**
Ian R. Falconer, Ph.D.	The research Report 13 "Oral Toxicity of Cylindrospermopsin: No Observed Adverse Effect Level Determination in Male Swiss Albino Mice" provides both consolidated data, and the individual observations, allowing detailed scrutiny. The methodology followed that recommended by the OECD. The project was carried out with the specific aim of allowing guideline values or reference dose determinations to be undertaken. It has been used for guideline determination for cylindrospermopsin in several countries.	
James S. Metcalf, Ph.D.	The research of Humpage and Falconer on the toxicity of cylindrospermopsin is the current "gold" standard in cyanotoxin toxicity research. The only potential issue is through the use of purified extracts, which may contain other bioactive compounds. However, similar research by Falconer was performed on microcystin toxicity in pigs using cyanobacterial extracts that have become the primary literature for the derivation of the WHO Guideline Values for microcystin-LR in drinking water. However, the study methodology is sound and dependable.	
Brett A. Neilan, Ph.D.	Yes. These studies are methodologically sound.	

CHARGE QUESTION 2: ***Are strengths and weaknesses of the study and the accompanying mode of action appropriately described? Please provide suggestions, if needed.***		
NAME	**COMMENT**	**RESPONSE**
Ian R. Falconer, Ph.D.	Yes, this is an appropriate assessment.	
James S. Metcalf, Ph.D.	The strengths and weaknesses and accompanying mode of action are adequately described.	
Brett A. Neilan, Ph.D.	Yes. The strengths and weaknesses of the studies are clearly described and the results are statistically supported. The physiological effects of cylindrospermopsin were described, however, a mode of action was not proposed for the toxin.	
CHARGE QUESTION 3: ***Do the results of this study represent the best available science and most appropriate toxicological endpoint for the basis of an oral RfD for cylindrospermopsin?***		
NAME	**COMMENT**	**RESPONSE**
Ian R. Falconer, Ph.D.	Yes, with the opportunity for detailed examination of the data in Report 13. Much more data are present in Report 13 than is described (or is necessary) in this review.	
James S. Metcalf, Ph.D.	The results of the study do represent the current best available science and most appropriate toxicological endpoints.	
Brett A. Neilan, Ph.D.	Yes. When considering all the published data, this study represents the best available science for the basis of an oral RfD for cylindrospermopsin. However, further detailed studies (acute and chronic) in both mice and human cell lines, if not organismic exposures, should be conducted in order to accurately predict safe drinking water guidelines for this cyanotoxin. Epidemiological studies in high-risk areas that experience contaminated water supplies should also be considered	
C. Calculation of RfD		
This Health Effects Support Document proposes an oral RfD for cylindrospermopsin based on the kidney weight data from the Humpage and Falconer studies (2002 and 2003) and supported by the hematological results identified in the Sukenik et al. 2006 study, and Reisner et al. 2004 (attached).		
CHARGE QUESTION 1: ***Is the calculation of the RfD for cylindrospermopsin clear and accurate? Please describe and provide suggestions, if needed.***		
NAME	**COMMENT**	**RESPONSE**
Ian R. Falconer, Ph.D.	Yes, the calculation follows the standard protocols for RfD determination.	

James S. Metcalf, Ph.D.	The calculation for RfD is clear and accurate.	
Brett A. Neilan, Ph.D.	The calculation is clear and accurate.	
CHARGE QUESTION 2: ***Has uncertainty (via uncertainty factors) been adequately accounted for in the derivation of the RfD? Please describe and provide suggestions, if needed.***		
NAME	**COMMENT**	**RESPONSE**
Ian R. Falconer, Ph.D.	The only component that has potential for variation is the value of safety factor UFd=3. To define this any closer will need further research, but is unlikely (in my view) to increase this factor. It may well be possible to reduce it.	
James S. Metcalf, Ph.D.	Not taking into account the potential for carcinogenicity, the uncertainty factors have been adequately accounted for.	
Brett A. Neilan, Ph.D.	Yes. Uncertainty has been adequately accounted for in the derivation of this relatively conservative RfD.	
Chapter 9 – Research Gaps		
CHARGE QUESTION 1: ***Have critical data gaps been identified and/or addressed for cylindrospermopsin? Please describe and provide suggestions, if needed.***		
NAME	**COMMENT**	**RESPONSE**
Ian R. Falconer, Ph.D.	These have been comprehensively identified. There is evidence pointing towards the answers for many of these research gaps, but they lack targeted replicated research, which will give clear information. The two outstanding gaps are related, one is the need for whole lifetime toxicology, which may show cumulative detrimental effects, and the other is rodent lifetime carcinogenicity trials. The human clinical data will always be an issue, as human poisoning events are (fortunately) infrequent and often occur in unusual locations, for example an offshore Australian island and a Brazilian dialysis clinic. It is possible that further study of the Palm Island clinical data, in the light of increasing experimental toxicology results, will allow a much more effective interpretation of the human hazard.	
James S. Metcalf, Ph.D.	Critical data gaps have been identified and addressed.	
Brett A. Neilan, Ph.D.	Yes. The manuscript identifies the major knowledge gaps for cylindrospermopsin research.	

General Questions		
CHARGE QUESTION 1: ***Is the document clear and understandable? Please describe and provide suggestions, if needed.***		
NAME	**COMMENT**	**RESPONSE**
Ian R. Falconer, Ph.D.	Yes.	
James S. Metcalf, Ph.D.	The document is clear and understandable.	
Brett A. Neilan, Ph.D.	Overall, the document is clear and understandable to the *specialist* reader. As mentioned previously, the manuscript could be significantly improved via the inclusion of introduction/conclusions paragraphs at the beginning/end of each chapter, with a final conclusions section at the end. Presently the reader is overwhelmed with data and is left to make their own conclusions, often requiring reference to the primary source of the data. The "synthesis and evaluation" sections are inadequate and arrive too late in the manuscript. A good review should not only summarize the available data. It should also synthesize, critically evaluate and translate that data for the target audience, which I assume will not be cyanotoxin specialists.	
CHARGE QUESTION 2: ***Are you aware of any additional data that should be addressed in the document? If so, please provide a reference.***		
NAME	**COMMENT**	**RESPONSE**
Ian R. Falconer, Ph.D.	Additional references are listed earlier and below [Specific Observations].	
James S. Metcalf, Ph.D.	Two additional references should be included. (1) Lindsay et al. (2006), Toxicon 48: 995-1001), who showed the potential for cyanobacterial LPS to alter the toxicological outcome of cylindrospermopsin in brine shrimp and (2) Wimmer et al. (2014; Harmful Algae 37: 203-206), describing new de-sulfo cylindrospermopsin variants in cyanobacteria.	
Brett A. Neilan, Ph.D.	No. The document contains a wealth of detailed data that adequately covers the research area.	
CHARGE QUESTION 3: ***Are you aware of any additional issues that should be addressed in the document? If so, please describe.***		
NAME	**COMMENT**	**RESPONSE**
Ian R. Falconer, Ph.D.	There is no reference to guideline values adopted by other nations.	

James S. Metcalf, Ph.D.	No additional issues should be addressed.	
Brett A. Neilan, Ph.D.	No. The document adequately addresses all issues pertinent to this research area.	

III. SPECIFIC OBSERVATIONS ON THE DOCUMENT				
NAME	**PG.**	**PAR.**	**COMMENT**	**RESPONSE**
Ian R. Falconer, Ph.D.	3	Chapter 2	Some comment on the high likelihood of raised DOC coincident with cyanobacterial blooms will be of value. In general, it is when water treatment plants are 'swamped' with organic material from a cyanobacterial bloom that toxins get through into the supply. See "Evidence of liver damage by toxin from a bloom of the blue-green alga Microcystis aeruginosa". Ian R.Falconer, Arthur M. Beresford and Maria T.C. Runnegar; Med.J.Aust 1983, 1, 511-514.	
	12	Section 3.2.1.2 para. 3, lines 5,6	This sentence is confusing, mixing the concept of activated carbon efficiency-which is a chemical and measurable property, the dose used, and the problems generated by dissolved organic carbon liberated during water blooms. Suggest expansion and re-writing, using the quoted references as sources.	
	14	Section 4.1, para. 1	Marine cyanobacteria grow as benthic organisms in shallow waters, often well out to sea, as well as free-floating water blooms. The most toxic, Lyngbya, grows below the low tide line. See "Cyanobacteria and Cyanobacterial Toxins" in Oceans and Human Health, eds Walsh P J et al., Chapter 15, pp 271-296. Academic Press, 2008.	
	17	Section 4.2.2	Drinking water. In the light of the very brief discussion here, reference to "Cyanobacterial Toxins of Drinking Water Supplies: Cylindrospermopsins and Microcystins", Ian Robert Falconer, CRC Press, Boca Raton, 2005 would be appropriate here. This section deserves a much more thorough approach, since it is the key issue for the whole review.	
	23	Section 7.1, para. 1,2	There is much more clinical data, but it is not published due to confidentiality provisions and the sensitive nature of the population, which was about 2,000 aboriginal persons, most of whom were exposed to the dam water supply. Preliminary examination of the cancer rates over the period 1982-1999, comparing the Palm Island	

III. SPECIFIC OBSERVATIONS ON THE DOCUMENT				
			population with comparable aboriginal population elsewhere, showed raised frequency of gastrointestinal, kidney and liver cancers, whereas the total cancer frequency was lower in the Palm Island population (unpublished). Copper sulphate precipitates rapidly from the water column. When used for cyanobacterial control, it is used at 1mg/L in the top metre of water, to provide an effective concentration of about 60 micrograms/L cupric ion. This been measured to disappear within 60-120 minutes. See "Cyanobacterial Toxins of Drinking Water Supplies: Cylindrospermopsins and Microcystins" Ian Robert Falconer, CRC Press, Boca Raton, 2005. There is no real possibility that the poisoning event was due to copper.	
	24	Para 3	Other routes of exposure. The toxicity seen in dialysis patients in Caruaru was probably due to the combined effects of microcystin and cylindrospermopsin, as there was nearly 10 times as much cylindrospermopsin in the filters as microcystin. From my examination of the liver histopathology, it was not possible to draw a finite conclusion, though it was not similar to microcystin damage seen in mice.	
	28		A relevant paper on reproductive and foetal toxicity is "Oral exposure to cylindrospermopsin in pregnant rats": Reproduction and foetal toxicity studies. Almeida et al., 2013, Toxicon 74:127-129. The results support the other studies, and conclude that the drinking water guideline value for cylindrospermopsin is safe (while they only quote proposed guidelines, these have been adopted with small variation, in Brazil, Australia, Canada.)	
	30	Last para., line 1	Re–examination of the data for the original report refers to mice C1M2 and C1M3 in the 240ug/kg/day dose group as having pathological changes in the kidney. Mouse C1M3, with the small groups of degenerating proximal tubule cells with leucocyte infiltration, had the highest kidney weight of any animal in the whole	

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			trial. Thus, the statement on page 59 of the report [Humpage, 2002] (ambiguously) refers to two mice with pathological kidney changes, not two sections of kidney from one mouse.	
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	44	Section 7.5.3	The analysis of the cyanobacterial toxin content of the dialysis filters showed 2.2 ug microcystin per g of filter material and 19.7 ug/g of cylindrospermopsin. However, there have been no studies on the toxicity of mixtures *in vivo* or *in vitro*. (Carmichael and Azevedo, 2001.)	
	45	Section 7.6.1	There is *in vivo* data for ribosomal separation from membranes of the endoplasmic reticulum Terao, 1994.	
	46	Para. 1, last line	The relevance of kidney effects in mice to human injury is very clear! The Palm island poisoning showed the most severe impacts on kidney function, water, electrolytes and protein loss led to the hospitalization of the children and several being put in intensive care, with electrolyte and protein replacement. 69% of the children required intravenous fluids, 12% required intravenous plasma protein solution. Without this support for kidney malfunction, several would have died. This human data strongly supports the experimental data on the impact of cylindrospermopsin on kidney function. For further *in vivo* mouse toxicology, see also Falconer et al., 1999, Environmental Toxicology 14:143-150.	
James S. Metcalf, Ph.D.	7	2	"specie" should be "species"	
	9	1	"aspects of the cyanobacteria" should be "aspects of cyanobacterial"	
	10	1	"cylindrospermopsin have shown" should be "cylindrospermopsin has been shown"	
	12	4	"overall structure of cylindrospermopsin" should be "overall structure of cylndrospermopsin variants"	

III. SPECIFIC OBSERVATIONS ON THE DOCUMENT				
	13	1	"cylindrospermopsin cells" should be "*Cylindrospermopsis* cells"	
	14	5	Biomass is the weight of a certain amount of cyanobacteria.	
	14	6	"drying the water" should be "drying particulates in the water"	
	15	3	"and" should not be italicized	
	15	4	It is possible that in the USGS survey, the *Aphanizomenon* and *Anabaena* in the blooms may produce cylindrospermopsin and not *Cylindrospermopsis*.	
	16	2	Blue-green algae is not used anymore and should be replaced by "cyanobacteria."	
	16	3	"specie" should be "species"	
	16	4	Blue-green algae is not used anymore and should be replaced by "cyanobacteria."	
	18	3	"toxin producing" should be "toxin-producing"	
	19	Table 5-1	The Funari and Testai paper is a review paper. The authors are referring to a study by Saker et al. (2004; Toxicon 43: 185-194) and this should be updated in the table.	
	19	1	"bluegreen" should be "blue-green"	
	19	1	It may be worth mentioning that BMAA and anatoxin-a have also been described in BGAS.	
	21	2	"to12" should be "to 12"	
	26	4	"organells" should be "organelles"	
	31	3	"later" should be "latter"	
	32	1	"extract and frozen" should be "extract was frozen"	
	34	1	Griffiths and Saker (2003) is a review and not a study.	
	41	3	"coloremetrically" should be "colorimetrically"	
Brett A. Neilan, Ph.D.	11	2	Cylindrospermopsin-producing cyanobacteria have not been reported in marine waters.	
	13	4	Gram not gram	
	16	1	cyrO not cyr0	
	17	2	The sentence "Deep water mixing and low light have been associated with an increase of *C. raciborskii* dominance, a toxin producing specie." is unclear.	

III. SPECIFIC OBSERVATIONS ON THE DOCUMENT				
	21	2	Transport - The cylindrospermopsin gene cluster contains an ORF designated *cyrK*, the product of which is most similar to sodium ion-driven multidrug and toxic compound extrusion proteins of the NorM family. It is hypothesized that CyrK is a cellular transporter for cylindrospermopsin based on this structural homology and its central location in the toxin biosynthesis gene cluster (see Mihali et al., 2008).	
	21	4	"Vertical mixing devices, bubblers, and other means of breaking down ~~de~~stratification have proven effective in controlling outbreaks and persistence of blooms in relatively small impoundments."	
	21	4	"The productivity and irradiance (the photosynthetically active radiation) of *C. raciborskii* was tested in a study in 2009 to determine how optical mixing depth will affect bloom production (O'Brien et al., 2009). The study demonstrated that an increase in the optical depth of mixing may increase *C. raciborskii* productivity." This implies that destratification is counterproductive for managing *C. raciborskii* blooms, which contradict the previous sentence.	
	24	3	"Most species of cyanobacteria are highly adaptable and inhabit freshwater and highly saline environments such as salt marches." This statement is misleading. While cyanobacteria as a phylum can grow in a vast range of habitats, different species/strains have different optimum growth conditions. Very few species/strains can thrive across a broad salt spectrum. Cylindrospermopsin-producers, for example, do not.	
	25	3	"and" and "spp." Should be not be italicized.	
	26	5	"In 2005, Oklahoma and the U.S. Army Corps of Engineers detected cylindrospermopsin at a maximum concentration of 1.6 µg/L." Was the bloom detected by or in Oklahoma?	
	27	2	"For the purpose of this document, we will focus on exposure by ingestion of drinking water contaminated with toxins and exposure by ingestion of water contaminated with toxins from exposure through fish consumption or recreational activities in freshwater sources." Fish should be changed to seafood, which also includes mussels, etc.	

III. SPECIFIC OBSERVATIONS ON THE DOCUMENT				
			Exposure via soil, edible plants and dietary supplements is also addressed in the manuscript.	
	27	4	"Exposure to high cyanotoxin concentrations in drinking water can result in acute/short term effects" is an understatement. Exposure to high levels of toxin can be deadly.	
	28	3	Remove "consumption" from the title.	
	29	1	Occurrence in dietary supplements has not been shown to be relevant to cylindrospermopsin, but is a potential hazard.	
	39	2	Why use bullet points here and nowhere else?	

III. INDIVIDUAL PEER REVIEWER COMMENTS

Review by:
Ian R. Falconer, Ph.D.

Peer Review Comments on EPA's Draft Document
"Health Effects Support Document for the Cyanobacterial Toxin Cylindrospermopsin"

Ian R. Falconer, Ph.D.
Water Quality Consultant; Hon. Visiting Fellow, Pharmacology,
University of Adelaide Medical Sciences, Adelaide, Australia

September 16, 2014

I. GENERAL IMPRESSIONS

This is a comprehensive and well assembled review of the field of cylindrospermopsin occurrence and toxicology. It is overall an accurate account of the available data, and the interpretation of the available information. Some specific points will be raised later, but there are no significant errors.

The presentation is systematic, but as a consequence of the approach, there is overlap of content. This can be consolidated, but the comprehensiveness of individual sections when viewed alone would be lost. If it is intended that chapters can be viewed as entities without reference to other chapters, then this comment can be ignored.

The conclusions are sound. The data from which the conclusions are drawn have a good level of internal consistency, with research groups from different laboratories, and countries, contributing to the overall conclusions. The issue of carcinogenicity of cylindrospermopsin will have to be addressed at more length when the crucial long-term carcinogenicity studies have been undertaken. Unfortunately, the cost of these studies has prevented their initiation up to the present time, and the focus, by necessity, has been on short-term and *in-vitro* research.

II. RESPONSE TO CHARGE QUESTIONS

Chapters 2, 5, and 6 of the Health Effects Support Document provide information on the chemical and physical properties, exposure, and the toxicokinetics of cylindrospermopsin.

1. Are you aware of any additional data that should be included in the document? If so, please provide.

Chapter 2.0, Page 13. Some comment on the high likelihood of raised DOC coincident with cyanobacterial blooms will be of value. In general, it is when water treatment plants are 'swamped' with organic material from a cyanobacterial bloom that toxins get through into the supply.

See: "Evidence of liver damage by toxin from a bloom of the blue-green alga Microcystis aeruginosa". Ian R. Falconer, Arthur M. Beresford, and Maria T.C. Runnegar; Med.J.Aust, 1983, 1: 511-514.

Chapter 4.0, Page 14, Section 4.1, Paragraph 1. Marine cyanobacteria grow as benthic organisms in shallow waters, often well out to sea, as well as free-floating water blooms. The most toxic, Lyngbya, grows below the low tide line.

See: "Cyanobacteria and Cyanobacterial Toxins" in Oceans and Human Health, eds. Walsh P J et al., Chapter 15, pp, 271-296. Academic Press, 2008.

Section 4.2.2 - drinking water. In the light of the very brief discussion here, reference to "Cyanobacterial Toxins of Drinking Water Supplies: Cylindrospermopsins and Microcystins," Ian Robert Falconer, CRC Press, Boca Raton, 2005, would be appropriate here. This section deserves a much more thorough approach, since it is the key issue for the whole review.

2. Is any of the information included in the document or conclusions incorrect, redundant or irrelevant? Please describe.

Section 3.2.1.2, Page 12, Paragraph 3, Lines 5 and 6. This sentence is confusing, mixing the concept of activated carbon efficiency-which is a chemical and measurable property, the dose used, and the problems generated by dissolved organic carbon liberated during water blooms. Suggest expansion and re-writing, using the quoted references as sources.

3. Please comment on the flow and continuity of these chapters and provide suggestions to enhance the utility of these chapters, if needed.

It is a large topic jump from occurrence of toxin to toxicokinetics. An introductory paragraph in Section 6 would smooth this and lead the reader from one to the other.

Chapter 7 - Hazard Identification. This chapter outlines the toxicity studies and the epidemiology, genotoxicity and mechanistic data. This chapter also includes the characterization of human health effects.

1. Are you aware of any additional critical studies for cylindrospermopsin that should be included in the document? If so, please provide.

Section 7.1, Paragraphs 1 and 2. There are much more clinical data, but it is not published due to confidentiality provisions and the sensitive nature of the population, which was about 2,000 aboriginal persons, most of whom were exposed to the dam water supply. Preliminary examination of the cancer rates over the period 1982-1999, comparing the Palm Island population with comparable aboriginal population elsewhere, showed raised frequency of gastrointestinal, kidney and liver cancers, whereas the total cancer frequency was lower in the Palm Island population (unpublished).

Copper sulphate precipitates rapidly from the water column. When used for cyanobacterial control, it is used at 1mg/L in the top metre of water, to provide an effective concentration of about 60 micrograms/L cupric ion. This has been measured to disappear within 60-120 minutes. See "Cyanobacterial Toxins of Drinking Water Supplies: Cylindrospermopsins and

Microcystins" Ian Robert Falconer, CRC Press, Boca Raton, 2005. There is no real possibility that the poisoning event was due to copper.

Page 24 - Other routes of exposure. The toxicity seen in dialysis patients in Caruaru was probably due to the combined effects of microcystin and cylindrospermopsin, as there was nearly 10 times as much cylindrospermopsin in the filters as microcystin. From my observation of the human liver histopathology, it was not possible to draw a finite conclusion, though it was not similar to microcystin damage seen in mice.

Page 28 - A relevant paper on reproductive and foetal toxicity is "Oral exposure to cylindrospermopsin in pregnant rats": Reproduction and Foetal Toxicity studies, Almeida et al., 2013, Toxicon 74: 127-129. The results support the other studies, and conclude that the drinking water guideline value for cylindrospermopsin is safe (while they only quote proposed guidelines, these have been adopted with small variation, in Brazil, Australia, Canada).

Page 30, last paragraph, last line. Re–examination of the original data for the report refers to mice C1M2 and C1M3 in the 240ug/kg/day dose group as having pathological changes in the kidney. Mouse C1M3, with the small groups of degenerating proximal tubule cells with leucocyte infiltration, had the highest kidney weight of any animal in the whole trial. Thus, the statement on page 59 of the report (ambiguously) refers to two mice with pathological kidney changes, not two sections from the same mouse.

Page 36, Paragraphs 1 and 2. The paper by Marie (quoted) concluded that the cell transforming activity of CYN indicates carcinogenic potential at very low concentrations *in vitro*. This with other studies quoted strengthens the case for careful investigation of carcinogenesis by CYN.

Page 44, Section 7.5.3. The analysis of the cyanobacterial toxin content of the dialysis filters showed 2.2 ug microcystin per gram of filter material and 19.7 ug/g of cylindrospermopsin. However, there have been no studies on the toxicity of mixtures *in vivo* or *in vitro*. (Carmichael and Azevedo, 2001).

Page 45, Section 7.6.1. There is *in vivo* data for ribosomal separation from membranes of the endoplasmic reticulum (Terao, 1994).

Page 46, Paragraph 1, last line. The relevance of kidney effects in mice to human injury is very clear! The Palm island poisoning showed the most severe impacts on kidney function; water, electrolytes and protein loss led to the hospitalization of the children and several being put in intensive care, with electrolyte and protein replacement. Sixty nine percent (69%) of the children required intravenous fluids, and 12% required intravenous plasma protein solution. Without this support for kidney malfunction, several would have died. This human data strongly supports the experimental data on the impact of cylindrospermopsin on kidney function. For further *in vivo* mouse toxicology, see also Falconer et al., 1999, Environmental Toxicology, 14:143-150.

2. Is any of the information included in the document incorrect, redundant or irrelevant? Please describe and provide suggestions, if needed.

See comments above.

3. Are the conclusions and critical discussions for cylindrospermopsin valid and scientifically defensible? Please describe and provide suggestions, if needed.

Yes, the overall assessment is correct and defensible.

Chapter 8 - Dose-Response Assessment. This chapter provides the dose-response assessment and the derivation of RfDs.

A. Data sufficiency

1. Is the conclusion that there are sufficient data to derive a reference dose (RfD) for cylindrospermopsin adequately justified? Please describe and provide suggestions, if needed.

The data presented show a good level of consistency between research groups and between animal strains. The overall organ sensitivity to the toxin shown in *in vivo* studies demonstrates kidney toxicity as a major concern, which fits with the extensive kidney damage seen in the human population, which was poisoned. Since the kidney weight data are the most sensitive, they are an effective parameter for reference dose calculation. There are sufficient data, backed up by the range of studies presented, and the detailed nature of the key study.

2. Have critical data gaps been identified and/or addressed for cylindrospermopsin? Please describe and provide suggestions, if needed.

There is a major gap in carcinogenicity assessment. No chronic rodent carcinogenicity study has been undertaken, notwithstanding the indicative in vitro research data. The cancer incidence in individuals identified as being hospitalized in the Palm Island poisoning, has not been obtained due to social issues. An overall cancer rate study showed increased cancers in specific organs, but no total increase in this population compared to similar groups.

B. Identification of the critical study

The Humpage and Falconer study (2002, 2003) was selected as the critical study for derivation of the RfD for cylindrospermopsin.

1. Is the study methodology sound? Please describe and provide suggestions, if needed.

The research Report 13 "Oral Toxicity of Cylindrospermopsin: No Observed Adverse Effect Level Determination in Male Swiss Albino Mice" provides both consolidated data, and the individual observations, allowing detailed scrutiny. The methodology followed that recommended by the OECD. The project was carried out with the specific aim of allowing guideline values or reference dose determinations to be undertaken. It has been used for guideline determination for cylindrospermopsin in several countries.

2. Are strengths and weaknesses of the study and the accompanying mode of action appropriately described? Please provide suggestions, if needed.

Yes, this is an appropriate assessment.

3. Do the results of this study represent the best available science and most appropriate toxicological endpoint for the basis of an oral RfD for cylindrospermopsin?

Yes, with the opportunity for detailed examination of the data in Report 13. Much more data are present in Report 13 than is described (or is necessary) in this review.

C. Calculation of RfD

This Health Effects Support Document proposes an oral RfD for cylindrospermopsin based on the kidney weight data from the Humpage and Falconer studies (2002 and 2003) and supported by the hematological results identified in the Sukenik et al. 2006 study, and Reisner et al. 2004 (attached).

1. Is the calculation of the RfD for cylindrospermopsin clear and accurate? Please describe and provide suggestions, if needed.

Yes, the calculation follows the standard protocols for RfD determination.

2. Has uncertainty (via uncertainty factors) been adequately accounted for in the derivation of the RfD? Please describe and provide suggestions, if needed.

The only component that has potential for variation is the value of safety factor UFd=3. To define this any closer will need further research, but is unlikely (in my view) to increase this factor. It may well be possible to reduce it.

Chapter 9 – Research Gaps

1. Have critical data gaps been identified and/or addressed for cylindrospermopsin? Please describe and provide suggestions, if needed.

These have been comprehensively identified. There is evidence pointing towards the answers for many of these research gaps, but they lack targeted replicated research, which will give clear information. The two outstanding gaps are related, one is the need for whole lifetime toxicology, which may show cumulative detrimental effects, and the other is rodent lifetime carcinogenicity trials.

The human clinical data will always be an issue, as human poisoning events are (fortunately) infrequent and often occur in unusual locations, for example an offshore Australian island and a Brazilian dialysis clinic. It is possible that further study of the Palm Island clinical data, in the light of increasing experimental toxicology results, will allow a much more effective interpretation of the human hazard.

General Questions

1. Is the document clear and understandable? Please describe and provide suggestions, if needed.

Yes.

2. Are you aware of any additional data that should be addressed in the document? If so, please provide a reference.

Additional references are listed earlier and below.

3. Are you aware of any additional issues that should be addressed in the document? If so, please describe.

There is no reference to guideline values adopted by other nations.

III. SPECIFIC OBSERVATIONS

Page	Paragraph	Comment or Question
3	Chapter 2	Some comment on the high likelihood of raised DOC coincident with cyanobacterial blooms will be of value. In general, it is when water treatment plants are 'swamped' with organic material from a cyanobacterial bloom that toxins get through into the supply. See "Evidence of liver damage by toxin from a bloom of the blue-green alga Microcystis aeruginosa". Ian R.Falconer, Arthur M. Beresford and Maria T.C. Runnegar; Med.J.Aust 1983, 1, 511-514.
12	Section 3.2.1.2 para. 3, lines 5, 6	This sentence is confusing, mixing the concept of activated carbon efficiency-which is a chemical and measurable property, the dose used, and the problems generated by dissolved organic carbon liberated during water blooms. Suggest expansion and re-writing, using the quoted references as sources.
14	Section 4.1, para. 1	Marine cyanobacteria grow as benthic organisms in shallow waters, often well out to sea, as well as free-floating water blooms. The most toxic, Lyngbya, grows below the low tide line. See "Cyanobacteria and Cyanobacterial Toxins" in Oceans and Human Health, eds Walsh P J et al., Chapter 15, pp 271-296. Academic Press, 2008.
17	Section 4.2.2	Drinking water. In the light of the very brief discussion here, reference to "Cyanobacterial Toxins of Drinking Water Supplies: Cylindrospermopsins and Microcystins", Ian Robert Falconer, CRC Press, Boca Raton, 2005 would be appropriate here. This section deserves a much more thorough approach, since it is the key issue for the whole review.
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Page	Paragraph	Comment or Question
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24	Para 3	Other routes of exposure. The toxicity seen in dialysis patients in Caruaru was probably due to the combined effects of microcystin and cylindrospermopsin, as there was nearly 10 times as much cylindrospermopsin in the filters as microcystin. From my examination of the liver histopathology, it was not possible to draw a finite conclusion, though it was not similar to microcystin damage seen in mice.
28		A relevant paper on reproductive and foetal toxicity is "Oral exposure to cylindrospermopsin in pregnant rats": Reproduction and foetal toxicity studies. Almeida et al., 2013, Toxicon 74:127-129. The results support the other studies, and conclude that the drinking water guideline value for cylindrospermopsin is safe (while they only quote proposed guidelines, these have been adopted with small variation, in Brazil, Australia, Canada.)
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36	Para. 1, 2	The paper by Marie (quoted) concluded that the cell transforming activity of CYN indicates carcinogenic potential at very low concentrations in vitro. This with other studies quoted strengthens the case for careful investigation of carcinogenesis by CYN.
44	Section 7.5.3	The analysis of the cyanobacterial toxin content of the dialysis filters showed 2.2 ug microcystin per g of filter material and 19.7 ug/g of cylindrospermopsin. However, there have been no studies on the toxicity of mixtures *in vivo* or *in vitro*. Carmichael and Azevedo, 2001.

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Review by:
James S. Metcalf, Ph.D.

Peer Review Comments on EPA's Draft Document
"Health Effects Support Document for the Cyanobacterial Toxin Cylindrospermopsin"

James S. Metcalf
Institute for Ethnomedicine

September 21, 2014

I. GENERAL IMPRESSIONS

The EPA document "Health Effects Support Document for Cyanobacterial Toxin Cylindrospermopsin" is an accurate, clear document addressing the effects of cylindrospermopsin in cells and mammals. Although it is clear and sound, there is a possibility to improve the document further, in order to maintain up to date and current information concerning the occurrence and toxicity of cylindrospermopsin and its variants. For example, in addition to cylindrospermopsin, 7-epicylindrospermopsin and deoxycylindrospermopsin, new variants of cylindrospermopsin, are being isolated and characterized (desulfo-cylindrospermopsin variants, Wimmer et al., 2014; Harmful Algae 37: 203-206), which although no toxicity data is available, do require mention, as they may contribute to the overall toxicity of cylindrospermopsin variants present in blooms and strains of cyanobacteria. Furthermore, although difficult, a large number of studies have examined the effects of cylindrospermopsin by use of extracts of cyanobacteria, with the possibility of synergistic effects with other bioactive compounds that may be present. It may be useful for strengthening the document by separating toxicity studies using extracts from those with purified cylindrospermopsin variants, along with some discussion concerning the different toxicity outcomes between such studies. In addition, it may be useful to include some invertebrate or plant based toxicity assays for risk assessment. This is because useful information concerning adverse cylindrospermopsin effects can be ascertained. For example, Lindsay et al. (2006; Toxicon 48: 995-1001) showed that co-exposure of LPS and cylindrospermopsin affected the subsequent LC_{50} values in brine shrimp, which has direct relevance for assessing human exposures to cylindrospermopsin in cyanobacterial blooms. However, the document as presented, does provide an accurate, up to date current risk assessment of cylindrospermopsin, based on current knowledge.

II. RESPONSE TO CHARGE QUESTIONS

Chapters 2, 5, and 6 of the Health Effects Support Document provide information on the chemical and physical properties, exposure, and the toxicokinetics of cylindrospermopsin.

1. Are you aware of any additional data that should be included in the document? If so, please provide.

It would be useful to include some mention and discussion of the study of Wimmer et al. (2014; Harmful Algae 37: 203-206) as they have described new de-sulfo variants of cylindrospermopsin, which to this reviewer, have not been discussed in prior documents pertaining to the adverse health effects of cylindrospermopsin.

2. Is any of the information included in the document or conclusions incorrect, redundant or irrelevant? Please describe.

There is no incorrect, redundant or irrelevant information in the document.

3. Please comment on the flow and continuity of these chapters and provide suggestions to enhance the utility of these chapters, if needed.

The chapters flow and continuity are fine.

Chapter 7 - Hazard Identification. This chapter outlines the toxicity studies and the epidemiology, genotoxicity and mechanistic data. This chapter also includes the characterization of human health effects.

4. Are you aware of any additional critical studies for cylindrospermopsin that should be included in the document? If so, please provide.

It may be useful to include some toxicity studies using invertebrates. For example, Lindsay et al. (2006) showed that co-exposure of cyanobacterial LPS and cylindrospermopsin affected the subsequent toxicity outcome, compared to the individual cyanotoxins. Therefore, such studies should be included as they may affect the subsequent human health risk assessment, especially as cyanobacterial LPS is considered to be always present in blooms, in addition to bacterial LPS from associated bacteria.

5. Is any of the information included in the document incorrect, redundant or irrelevant? Please describe and provide suggestions, if needed.

There is no incorrect, redundant or irrelevant information in the document.

6. Are the conclusions and critical discussions for cylindrospermopsin valid and scientifically defensible? Please describe and provide suggestions, if needed.

The conclusions and critical discussion for cylindrospermopsin are valid and scientifically defensible.

Chapter 8 - Dose-Response Assessment. This chapter provides the dose-response assessment and the derivation of RfDs.

A. Data sufficiency

1. Is the conclusion that there are sufficient data to derive a reference dose (RfD) for cylindrospermopsin adequately justified? Please describe and provide suggestions, if needed.

In terms of mammals, the authors have used all of the available toxicity data. The only potential issue arises from the fact that only one study used purified CYN, whereas the remainder used

cell-free extracts. This latter point may have issues concerning potential synergistic or additive effects from other bioactive compounds present within the extract. However, no other mammalian data are currently available.

2. Have critical data gaps been identified and/or addressed for cylindrospermopsin? Please describe and provide suggestions, if needed.

There are no further critical gaps.

B. Identification of the critical study

The Humpage and Falconer study (2002, 2003) was selected as the critical study for derivation of the RfD for cylindrospermopsin.

1. Is the study methodology sound? Please describe and provide suggestions, if needed.

The research of Humpage and Falconer on the toxicity of cylindrospermopsin is the current "gold" standard in cyanotoxin toxicity research. The only potential issue is through the use of purified extracts, which may contain other bioactive compounds. However, similar research by Falconer was performed on microcystin toxicity in pigs using cyanobacterial extracts that have become the primary literature for the derivation of the WHO Guideline Values for microcystin-LR in drinking water. However, the study methodology is sound and dependable.

2. Are strengths and weaknesses of the study and the accompanying mode of action appropriately described? Please provide suggestions, if needed.

The strengths and weaknesses and accompanying mode of action are adequately described.

3. Do the results of this study represent the best available science and most appropriate toxicological endpoint for the basis of an oral RfD for cylindrospermopsin?

The results of the study do represent the current best available science and most appropriate toxicological endpoints.

C. Calculation of RfD

This Health Effects Support Document proposes an oral RfD for cylindrospermopsin based on the kidney weight data from the Humpage and Falconer studies (2002 and 2003) and supported by the hematological results identified in the Sukenik et al. 2006 study, and Reisner et al. 2004 (attached).

1. Is the calculation of the RfD for cylindrospermopsin clear and accurate? Please describe and provide suggestions, if needed.

The calculation for RfD is clear and accurate.

2. Has uncertainty (via uncertainty factors) been adequately accounted for in the derivation of the RfD? Please describe and provide suggestions, if needed.

Not taking into account the potential for carcinogenicity, the uncertainty factors have been adequately accounted for.

Chapter 9 – Research Gaps

1. Have critical data gaps been identified and/or addressed for cylindrospermopsin? Please describe and provide suggestions, if needed.

Critical data gaps have been identified and addressed.

General Questions

1. Is the document clear and understandable? Please describe and provide suggestions, if needed.

The document is clear and understandable.

2. Are you aware of any additional data that should be addressed in the document? If so, please provide a reference.

Two additional references should be included. (1) Lindsay et al. (2006; Toxicon 48: 995-1001), who showed the potential for cyanobacterial LPS to alter the toxicological outcome of cylindrospermopsin in brine shrimp and (2)Wimmer et al. (2014; Harmful Algae 37: 203-206), describing new de-sulfo cylindrospermopsin variants in cyanobacteria.

3. Are you aware of any additional issues that should be addressed in the document? If so, please describe.

No additional issues should be addressed.

III. SPECIFIC OBSERVATIONS

Provide specific observations or comments on the study report mentioning page and paragraph (expand table if needed).

Page	Paragraph	Comment or Question
7	2	"specie" should be "species"
9	1	"aspects of the cyanobacteria" should be "aspects of cyanobacterial"
10	1	"cylindrospermopsin have shown" should be "cylindrospermopsin has been shown"
12	4	"overall structure of cylindrospermopsin" should be "overall structure of cylndrospermopsin variants"

Page	Paragraph	Comment or Question
13	1	"cylindrospermopsin cells" should be "*Cylindrospermopsis* cells"
14	5	Biomass is the weight of a certain amount of cyanobacteria.
14	6	"drying the water" should be "drying particulates in the water"
15	3	"and" should not be italicized
15	4	It is possible that in the USGS survey, the *Aphanizomenon* and *Anabaena* in the blooms may produce cylindrospermopsin and not *Cylindrospermopsis*.
16	2	Blue-green algae is not used anymore and should be replaced by "cyanobacteria."
16	3	"specie" should be "species"
16	4	Blue-green algae is not used anymore and should be replaced by "cyanobacteria."
18	3	"toxin producing" should be "toxin-producing"
19	Table 5-1	The Funari and Testai paper is a review paper. The authors are referring to a study by Saker et al. (2004; Toxicon 43: 185-194) and this should be updated in the table.
19	1	"bluegreen" should be "blue-green"
19	1	It may be worth mentioning that BMAA and anatoxin-a have also been described in BGAS.
21	2	"to12" should be "to 12"
26	4	"organells" should be "organelles"
31	3	"later" should be "latter"
32	1	"extract and frozen" should be "extract was frozen"
34	1	Griffiths and Saker (2003) is a review and not a study.
41	3	"coloremetrically" should be "colorimetrically"

Review by:
Brett A. Neilan, Ph.D.

Peer Review Comments on EPA's Draft Document
"Health Effects Support Document for the Cyanobacterial Toxin Cylindrospermopsin"

Brett A. Neilan, Ph.D.
The University of New South Wales

September 17, 2014

I. GENERAL IMPRESSIONS

Overall, the information in the document is accurate and clearly presented. The final conclusions are sound, however the manuscript could be improved by further critical evaluation of the literature throughout. The main flaws of this manuscript are the inconsistent level of detail and the general lack of synthesis of the toxicological studies cited. For example, Chapters 2-5 contain a large volume of superfluous and very general information on cyanobacteria and should be streamlined to include information specific to cylindrospermopsin and its producers. In contrast, Chapters 6-7, dealing with cynlindrospermopsin toxicology, contain very detailed information but lack synthesis and a critical review of this data. These omissions are partially addressed in Section 7.6, however, the brief summary paragraphs provided are inadequate and the disjointed structure employed disrupts the flow of the manuscript, which then necessitates repetition of the information.

II. RESPONSE TO CHARGE QUESTIONS

Chapters 2, 5, and 6 of the Health Effects Support Document provide information on the chemical and physical properties, exposure, and the toxicokinetics of cylindrospermopsin.

1. ***Are you aware of any additional data that should be included in the document? If so, please provide.***

These sections are comprehensive. I am unaware of any additional data that should be included.

2. ***Is any of the information included in the document or conclusions incorrect, redundant or irrelevant? Please describe.***

Chapter 2 should be streamlined to focus more on cylindrospermopsin-producing cyanobacteria. Chapter 5 contains largely irrelevant information on the occurrence of cyanotoxins in dietary supplements. This section should be condensed since cylindrospermopsin is a potential but non-specific risk. To my knowledge, there have been no reports of cylindrospermopsin contamination in such health products. The negative results of several studies suggest that the risk of cylindrospermopsin poisoning by this route is very low.

3. ***Please comment on the flow and continuity of these chapters and provide suggestions to enhance the utility of these chapters, if needed.***

The flow and continuity of Chapters 2 and 5 are acceptable. Chapter 6 lacks proper structure, that is, there is no introduction/overview or discussion. The chapter is simply a summary of the results of various animal studies. Like most of the other toxicology sections, it reads like a mosaic of scientific abstracts. The reader is left to wade through the data and formulate their own conclusions.

Chapter 7 - Hazard Identification. This chapter outlines the toxicity studies and the epidemiology, genotoxicity and mechanistic data. This chapter also includes the characterization of human health effects.

1. ***Are you aware of any additional critical studies for cylindrospermopsin that should be included in the document? If so, please provide.***

This section is comprehensive. I am unaware of any additional data that should be included.

2. ***Is any of the information included in the document incorrect, redundant or irrelevant? Please describe and provide suggestions, if needed.***

All the information in Chapter 7 appears to be accurate and relevant.

3. ***Are the conclusions and critical discussions for cylindrospermopsin valid and scientifically defensible? Please describe and provide suggestions, if needed.***

The conclusions and critical discussions are valid and scientifically defensible. However, further acknowledgement of the limitations of the published studies used to construct this section should be included. The lack of standardization of methods, controls, animal species, toxins, and sample sizes make it difficult to compare these previous studies. This has been a serious problem in cyanotoxin research as in other aspects of environmental toxicology and should be acknowledged in this section of the draft EPA document in order to make any reader/user of the information aware of these limitations.

Chapter 8 - Dose-Response Assessment. This chapter provides the dose-response assessment and the derivation of RfDs.

A. Data sufficiency

1. ***Is the conclusion that there are sufficient data to derive a reference dose (RfD) for cylindrospermopsin adequately justified? Please describe and provide suggestions, if needed.***

The lack of human case studies makes it difficult to assess the effect of cylindrospermopsin on humans. All risk assessments must therefore be based on data obtained from animal experiments. However, relatively few comparable animal toxicology studies have been conducted for cylindrospermopsin. In the present manuscript, the RfD for humans is based on two studies (Humpage and Falconer 2002, 2003). These studies relied on a small sample size (10 mice). The results were statistically significant and the proposed No Observed Adverse Effect Level

(NOAEL) for mice is adequately justified. Extrapolating these results to humans is complicated and numerous factors must be considered, including differences in toxin transport and metabolism, as well as relative body size and mode of introduction. It is therefore impossible to accurately predict the Equivalent NOAEL for humans based on these studies alone. Further toxicological and pharmacokinetic animal studies are required to enable accurate scaling and estimation of NOAEL before a confident RfD value can be put forward.

2. Have critical data gaps been identified and/or addressed for cylindrospermopsin? Please describe and provide suggestions, if needed.

Yes. The manuscript identifies the major knowledge gaps for cylindrospermopsin research.

B. Identification of the critical study

The Humpage and Falconer study (2002, 2003) was selected as the critical study for derivation of the RfD for cylindrospermopsin.

1. Is the study methodology sound? Please describe and provide suggestions, if needed.

Yes. These studies are methodologically sound.

2. Are strengths and weaknesses of the study and the accompanying mode of action appropriately described? Please provide suggestions, if needed.

Yes. The strengths and weaknesses of the studies are clearly described and the results are statistically supported. The physiological effects of cylindrospermopsin were described, however, a mode of action was not proposed for the toxin.

3. Do the results of this study represent the best available science and most appropriate toxicological endpoint for the basis of an oral RfD for cylindrospermopsin?

Yes. When considering all the published data, this study represents the best available science for the basis of an oral RfD for cylindrospermopsin. However, further detailed studies (acute and chronic) in both mice and human cell lines, if not organismic exposures, should be conducted in order to accurately predict safe drinking water guidelines for this cyanotoxin. Epidemiological studies in high-risk areas that experience contaminated water supplies should also be considered.

C. Calculation of RfD

This Health Effects Support Document proposes an oral RfD for cylindrospermopsin based on the kidney weight data from the Humpage and Falconer studies (2002 and 2003) and supported by the hematological results identified in the Sukenik et al. 2006 study, and Reisner et al. 2004 (attached).

1. Is the calculation of the RfD for cylindrospermopsin clear and accurate? Please describe and provide suggestions, if needed.

The calculation is clear and accurate.

2. Has uncertainty (via uncertainty factors) been adequately accounted for in the derivation of the RfD? Please describe and provide suggestions, if needed.

Yes. Uncertainty has been adequately accounted for in the derivation of this relatively conservative RfD.

Chapter 9 – Research Gaps

1. Have critical data gaps been identified and/or addressed for cylindrospermopsin? Please describe and provide suggestions, if needed.

Yes. The manuscript identifies the major knowledge gaps for cylindrospermopsin research.

General Questions

1. Is the document clear and understandable? Please describe and provide suggestions, if needed.

Overall, the document is clear and understandable to the *specialist* reader. As mentioned previously, the manuscript could be significantly improved via the inclusion of introduction/conclusions paragraphs at the beginning/end of each chapter, with a final conclusions section at the end. Presently the reader is overwhelmed with data and is left to make their own conclusions, often requiring reference to the primary source of the data. The "synthesis and evaluation" sections are inadequate and arrive too late in the manuscript.

A good review should not only summarize the available data. It should also synthesize, critically evaluate and translate that data for the target audience, which I assume will not be cyanotoxin specialists.

2. Are you aware of any additional data that should be addressed in the document? If so, please provide a reference.

No. The document contains a wealth of detailed data that adequately covers the research area.

3. Are you aware of any additional issues that should be addressed in the document? If so, please describe.

No. The document adequately addresses all issues pertinent to this research area.

III. SPECIFIC OBSERVATIONS

Page	Paragraph	Comment or Question
11	2	Cylindrospermopsin-producing cyanobacteria have not been reported in marine waters.

Page	Paragraph	Comment or Question
13	4	Gram not gram
16	1	cyrO not cyr0
17	2	The sentence "Deep water mixing and low light have been associated with an increase of *C. raciborskii* dominance, a toxin producing specie." is unclear.
21	2	Transport - The cylindrospermopsin gene cluster contains an ORF designated *cyrK*, the product of which is most similar to sodium ion-driven multidrug and toxic compound extrusion proteins of the NorM family. It is hypothesized that CyrK is a cellular transporter for cylindrospermopsin based on this structural homology and its central location in the toxin biosynthesis gene cluster (see Mihali et al., 2008).
21	4	"Vertical mixing devices, bubblers, and other means of breaking down ~~de~~stratification have proven effective in controlling outbreaks and persistence of blooms in relatively small impoundments."
21	4	"The productivity and irradiance (the photosynthetically active radiation) of *C. raciborskii* was tested in a study in 2009 to determine how optical mixing depth will affect bloom production (O'Brien et al., 2009). The study demonstrated that an increase in the optical depth of mixing may increase *C. raciborskii* productivity." This implies that destratification is counterproductive for managing *C. raciborskii* blooms, which contradict the previous sentence.
24	3	"Most species of cyanobacteria are highly adaptable and inhabit freshwater and highly saline environments such as salt marches." This statement is misleading. While cyanobacteria as a phylum can grow in a vast range of habitats, different species/strains have different optimum growth conditions. Very few species/strains can thrive across a broad salt spectrum. Cylindrospermopsin-producers, for example, do not.
25	3	"and" and "spp." Should be not be italicized.
26	5	"In 2005, Oklahoma and the U.S. Army Corps of Engineers detected cylindrospermopsin at a maximum concentration of 1.6 µg/L." Was the bloom detected by or in Oklahoma?
27	2	"For the purpose of this document, we will focus on exposure by ingestion of drinking water contaminated with toxins and exposure by ingestion of water contaminated with toxins from exposure through fish consumption or recreational activities in freshwater sources." Fish should be changed to seafood, which also includes mussels, etc. Exposure via soil, edible plants and dietary supplements is also addressed in the manuscript.
27	4	"Exposure to high cyanotoxin concentrations in drinking water can result in acute/short term effects" is an understatement. Exposure to high levels of toxin can be deadly.
28	3	Remove "consumption" from the title.
29	1	Occurrence in dietary supplements has not been shown to be relevant to cylindrospermopsin, but is a potential hazard.

Page	Paragraph	Comment or Question
39	2	Why use bullet points here and nowhere else?